EXHIBIT C

The Lancet article published August 10, 2002, entitled "Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial"

Articles

Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial

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Summary

Background Preclinical studies have established that implantation of bone marrow-mononuclear cells, including endothelial progenitor cells, into ischaemic limbs increases collateral vessel formation. We investigated efficacy and safety of autologous implantation of bone marrow-mononuclear cells in patients with ischaemic limbs because of peripheral arterial disease.

Methods We first did a pilot study, in which 25 patients (group A) with unilateral ischaemia of the leg were injected with bone marrow-mononuclear cells into the gastrocnemius of the ischaemic limb and with saline into the less ischaemic limb. We then recruited 22 patients (group B) with bilateral leg ischaemia, who were randomly injected with bone marrow-mononuclear cells in one leg and peripheral blood-mononuclear cells in the other as a control. Primary outcomes were safety and feasibility of treatment, based on ankle-brachial index (ABI) and rest pain, and analysis was per protocol.

Findings Two patients were excluded from group B after randomisation. At 4 weeks in group B patients, ABI was significantly improved in legs injected with bone marrow-mononuclear cells compared with those injected with peripheral blood-mononuclear cells (difference 0·09 [95% CI 0·06–0·11]; p<0·0001). Similar improvements were seen for transcutaneous oxygen pressure (13 [9–17]; p<0·0001), rest pain (–0·85 [–1·6 to –0·12]; p=0·025), and pain-free walking time (1·2 [0·7–1·7]; p=0·0001). These improvements were sustained at 24 weeks. Similar improvements were seen in group A patients. Two patients in group A died after myocardial infarction unrelated to treatment.

Interpretation Autologous implantation of bone marrow-mononuclear cells could be safe and effective for achievement of therapeutic angiogenesis, because of the natural ability of marrow cells to supply endothelial progenitor cells and to secrete various angiogenic factors or cytokines.

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Introduction

Results of preclinical studies have shown that angiogenic growth factors promote development of collateral arteries, which is called therapeutic angiogenesis.[1] Angiogenesis can be achieved either by use of growth-factors or genes encoding these proteins. Limited clinical data from protein-delivery and gene-delivery trials suggests that both approaches are safe. However, a great deal more clinical experience is needed to resolve safety concerns such as potentiation of pathological angiogenesis (eg, malignancy) and so-called bystander effects of delivered factors (eg, effects on kidney or atheroma).[2] In view of the enclosure of formed mature vessels with periendothelial matrix and pericyte, smooth-muscle cells, or both, treatment with various angiogenic growth factors might be preferable in future treatments.[3]

Endothelial progenitor cells in the $CD34^+$ stem-cell fraction of adult human peripheral blood take part in postnatal neovascularisation after mobilisation from bone marrow.[4,5] Although $CD34^-$ cells enhance $CD34^+$

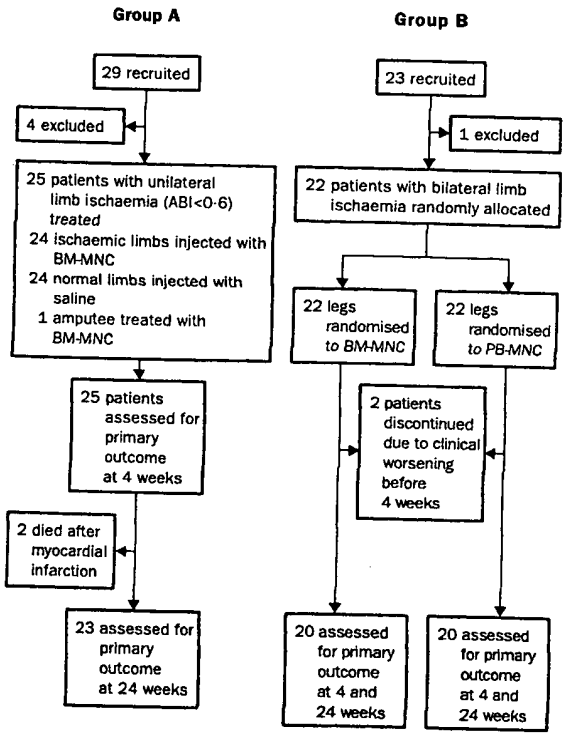

Figure 1: **Trial profile**
BM-MNC=bone marrow-mononuclear cells; PB-MNC=peripheral blood-mononuclear cells.

	All patients (n=45)	Group A (n=25)	Group B (n=20)
Age (years, mean [SD])	66 (12)	67 (13)	69 (11)
Sex			
Male	38 (84%)	20 (80%)	18 (90%)
Female	7 (16%)	5 (20%)	2 (10%)
Previous treatment			
PTA	5 (11%)	3 (12%)	2 (10%)
Bypass graft	24 (53%)	13 (52%)	11 (55%)
PTA and bypass graft	8 (18%)	4 (16%)	4 (20%)
Ischaemic status			
Non-healing ulcer	10 (22%)	6 (24%)	4 (20%)
Gangrene	18 (40%)	8 (32%)	10 (50%)
Disorders			
Hypertension	32 (71%)	18 (72%)	14 (70%)
Hyperlipidaemia	19 (42%)	8 (32%)	11 (55%)
Diabetes	31 (69%)	18 (72%)	13 (65%)
Chronic renal failure	5 (11%)	5 (20%)	0
ABI (mean [SD])			
BM-MNC implanted limb	0·35 (0·14)	0·34 (0·16)	0·37 (0·12)
PB-MNC or saline implanted limb	0·61 (0·19)	0·71 (0·08)	0·40 (0·11)
TcO_2 (mm Hg, mean [SD])			
BM-MNC implanted limb	28 (10)	28 (11)	29 (9)
PB-MNC or saline implanted limb	44 (12)	56 (9)	31 (9)
Pain-free walking time (min, mean [SD])	1·3 (0·5)	1·6 (0·8)	0·8 (0·3)
Implanted cell number (mean [SD])			
BM-MNC (10^9 cells)	1·6 (0·6)	1·6 (0·6)	1·5 (0·6)
CD34 in BM-MNC (10^7 cells)	3·7 (1·8)	3·9 (2·2)	3·5 (1·3)
PB-MNC (10^9 cells)	0·003 (0·001)	0·003 (0·001)	1·5 (0·6)

Data are number (%) unless otherwise indicated. PTA=percutaneous angioplasty. ABI=ankle-brachial pressure index. BM-MNC=bone marrow-mononuclear cells. PB-MNC=peripheral blood-mononuclear cells. TcO_2=transcutaneous oxygen pressure.

Table 1: **Patients' characteristics**

cell-mediated angiogenesis,[4] the underlying mechanism remains undefined. We,[6] and Kalka and colleagues,[7] noted that mononuclear cells from adult human peripheral or cord blood improved capillary density in hindlimb ischaemia. Marrow stromal cells have many of the characteristics of stem cells for mesenchymal tissues, and also secrete many angiogenic cytokines,[8–10] raising the possibility that marrow implantation into ischaemic limbs could enhance angiogenesis by supplying endothelial progenitor cells and angiogenic cytokines or factors.

Consistent with this hypothesis, we have shown in animals that bone marrow-mononuclear cell implantation into ischaemic limbs[11] or myocardium[12] promotes collateral vessel formation, with incorporation of endothelial progenitor cells into new capillaries, and that local concentrations of angiogenic factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF], and angiopoietin-1) or angiogenic cytokines (interleukin 1β and tumour necrosis factor α) were increased in implanted tissues. Neither tissue injury by inflammatory cytokines released from injected cells nor differentiation into other lineage cells, such as osteoblasts or fibroblasts, was noted in implanted ischaemic tissues.

On the basis of these results in animals, we started a clinical trial to test cell therapy with autologous bone marrow-mononuclear cells in patients with ischaemic limbs.

Patients and methods

Patients

Patients qualified for marrow implantation if they had chronic limb ischaemia, including rest pain, non-healing ischaemic ulcers, or both, and were not candidates for non-surgical or surgical revascularisation.[13] Requisite haemodynamic deficits included resting ankle-brachial pressure index (ABI) less than 0·6 in the affected limb on two consecutive examinations done at least 1 week apart. We excluded patients with poorly controlled diabetes mellitus (HbA_{1c} >6·5% and proliferative retinopathy) or with evidence of malignant disorder during the past 5 years. We obtained written informed consent from all patients. Ethics committees of participating universities approved the protocol.

Procedures

Primary outcomes were safety and feasibility of treatment, defined as improvements in ABI, transcutaneous oxygen pressure (TcO_2), and rest pain. Since there are no published data on this particular procedure, we could not precalculate numbers of patients needed for the study. We therefore did a pilot study, for which we recruited 29 patients (group A) with unilateral limb ischaemia, and to 25 of these we gave active treatment (implantation of bone marrow-mononuclear cells) into the more ischaemic leg (ABI <0·6) and control treatment (saline injection) into the opposite, less ischaemic leg (ABI >0·6). On the basis of ABI values at week 4, statistical power was more than 90% (two-sided α 0·05) for ten patients. We considered 20 patients to be a

	Group A (unmasked, n=25)				Group B (randomised, double-blind, n=20)			
	Change from baseline		Difference (95% CI)	p	Change from baseline		Difference (95% CI)	p
	BM-MNC	Saline			BM-MNC	PB-MNC		
Variable (mean [SD])								
ABI								
4 weeks	*0·13 (0·1)*	*0·01 (0·02)*	*0·12 (0·09 to 0·16)*	*<0·0001*	*0·1 (0·05)*	*0·02 (0·02)*	*0·09 (0·06 to 0·11)*	*<0·0001*
24 weeks	0·11 (0·1)	–0·01 (0·02)	0·11 (0·07 to 0·15)	<0·0001	0·1 (0·05)	0·02 (0·03)	0·09 (0·06 to 0·12)	<0·0001
TcO_2 (mm Hg)								
4 weeks	19 (12)	0·5 (1·4)	17 (12 to 22)	<0·0001	17·4 (9·5)	4·6 (3·5)	13 (9 to 17)	<0·0001
24 weeks	18 (11)	1·1 (2·6)	16 (11 to 21)	<0·0001	16·6 (9·9)	4·8 (2·8)	12 (7 to 16)	<0·0001
Rest pain (+4 to 0)*								
4 weeks	*–2·6 (1·1)*	*–0·25 (0·7)*	*–2·3 (–3 to –1·8)*	*<0·0001*	*–2·2 (1·1)*	*–1·4 (0·7)*	*–0·85 (–1·6 to –0·12)*	*0·025*
24 weeks	–2·6 (0·9)	+0·25 (0·7)	–2·8 (–3 to –2)	<0·0001	–2·4 (0·8)	–1·4 (1·1)	–1·0 (–1·7 to –0·32)	0·0061
New collateral (+3 to 0)†								
4 weeks	1 (1)	0 (0)	1 (0·6 to 1·5)	<0·0001	1·1 (1)	0·3 (0·6)	0·85 (0·3 to 1·4)	0·0025
Pain-free walking time (min)								
4 weeks	..	..	3·4 (2·7 to 4·2)	<0·0001	..	..	1·2 (0·7 to 1·7)	0·0001
24 weeks	..	..	3·5 (3·0 to 4·2)	<0·0001	..	..	1·4 (0·9 to 1·8)	<0·0001

BM-MNC=bone marrow-mononuclear cells. PB-MNC=peripheral blood-mononuclear cells. ABI=ankle-brachial pressure index. TcO_2=transcutaneous oxygen pressure. *Rest pain scale: +4, severe pain unresolved with paracetamol or non-steroidal anti-inflammatory drugs (NSAID); +3, moderate pain NSAID necessary; +2, slight pain NSAID unnecessary; +1, very slight pain; 0, completely resolved. †Angiographic scores for new collateral vessel formation: +3, rich; +2, moderate; +1, slight; 0, no change.

Table 2: **Differences in primary outcome between groups**

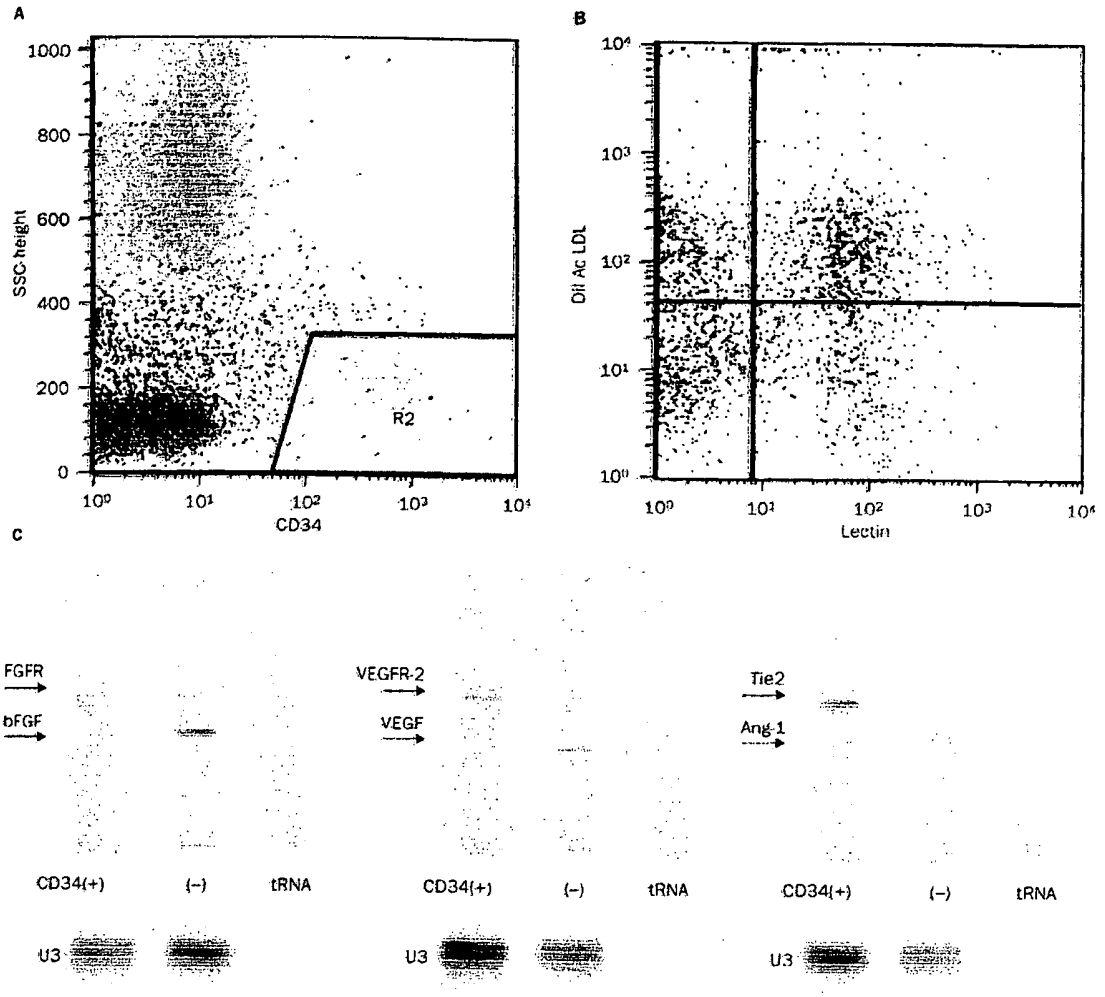

Figure 2: **Identification of endothelial lineage cells and expression of angiogenic factors in bone marrow-mononuclear cells**
Fluorescence activated cell sorting shows CD34⁺ fraction (R2 area in A) and endothelial lineage cells (upper right in B). (C) RNase protection assay of basic fibroblast growth factor (bFGF) and its receptor FGFR-1, vascular endothelial growth factor (VEGF) and its receptor VEGFR-2, and angiopoietin-1 (Ang-1) and its receptor Tie2 in CD34⁺ and CD34⁻ fractions. Same fractions were analysed by northern blotting with a U3 rRNA probe as an internal standard. Figure represents samples from eight different patients in group A.

practical number to detect statistical differences between treatments, thus we recruited a further 23 patients (group B) whose bilateral legs were ischaemic (ABI <0·6; figure 1 and table 1).

We randomly allocated 22 patients in group B to implantation of either bone marrow-mononuclear cells (active treatment) or peripheral blood-mononuclear cells (control) into the right or left ischaemic leg in a simultaneous within-patient trial fashion. Two patients discontinued before 4 weeks (figure 1). We considered peripheral blood-mononuclear cells to be a more appropriate cell control than saline, since peripheral blood is partly contaminated during the marrow aspiration procedure (about 10% of marrow cells), and the number of CD34 cells, including endothelial progenitor cells, is about 500-fold lower in peripheral blood-mononuclear cells than in bone marrow-mononuclear cells.

Randomisation of group B patients was done before treatment under the direction of a steering committee (Cardiovascular Center, Kansai Medical University), and was computer-generated with SAS (version 6.12), with a block size of four.[14] Allocation was done by central fax, and the sequence was concealed until interventions were assigned. Investigators who gave the interventions and assessed outcomes were masked to group assignment apart from chief investigators in participating hospitals. Injections of concentrated bone marrow-mononuclear cells or peripheral blood-mononuclear cells were identical in appearance. First recruitment started from Jan 25, 2000, and follow-up was 24 weeks.

While patients were under general anaesthesia, we aspirated marrow cells (about 500 mL) from the ileum, and gathered these into plastic bags containing heparin. We sorted mononuclear cells on a CS3000-Plus blood-cell separator (Baxter, Deerfield, USA) to 95% purity[15] and concentrated them to a final volume of about 30 mL immediately before aspiration. We implanted cells about 3 h after marrow aspiration by intramuscular injection into the gastrocnemius of ischaemic legs ($2·7\times10^9$ to $0·7\times10^9$ cells in group A and $2·8\times10^9$ to $0·88\times10^9$ cells in

group B). On the basis of May-Giemsa staining (done in 34 participants; 14 in group A, 20 in B), the sorted bone marrow-mononuclear cells contained lymphocytoid cells (mean 69%, SD 13), erythroblasts (8%, 4), monocytoid cells (8%, 4), and granulocytes (15%, 5). Total injection volume was about 30 mL, and we implanted about 0·75 mL of bone marrow-mononuclear cells into each injection site, with a 3×3 cm grid and 26-gauge needle (40 sites, 1·5 cm deep). Baseline ABI in the 45 most ischaemic limbs showed significant positive correlations with baseline pain-free walking time (r=0·69, p=0·028) and TcO_2 (r=0·54, p=0·032), suggesting the validity of these variables to assess status of limb ischaemia.

We followed up patients every week for 4 weeks after implantation and every 4 months thereafter, and monitored ABI, TcO_2, and pain-free walking time (treadmill at 3 km/h with no incline).[16] We used the criteria of Rutherford and colleagues[16] to assess limb status. To measure ABI, we established doppler-derived arterial segmental pressures on the ankle and brachium with a standard adult cuff, and indexed ankle systolic pressure against brachial systolic pressure (normal range >1·0).[17] We judged an increase in ABI of more than 0·1 an improvement, according to standard assessment of interventional therapy for peripheral artery diseases.[17] We measured TcO_2 with an oxymonitor (PO-850, Sumitomo-Hightechs, Tokyo, Japan). After

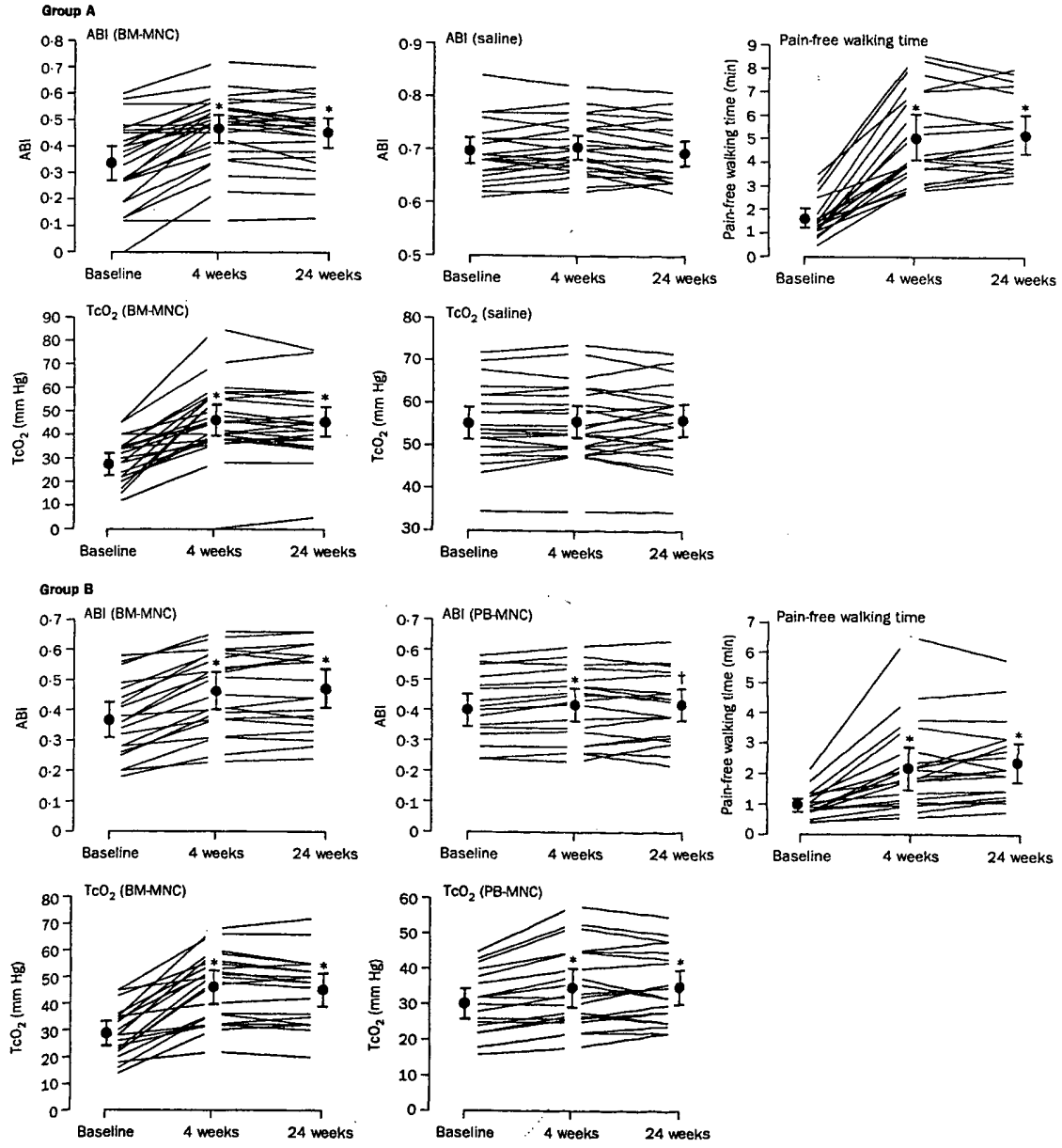

Figure 3: **Blood flow in limbs injected with bone marrow-mononuclear cells in group A and group B**
Results are shown as mean and 95% CI. *p<0·0001, †p=0·012 versus baseline.

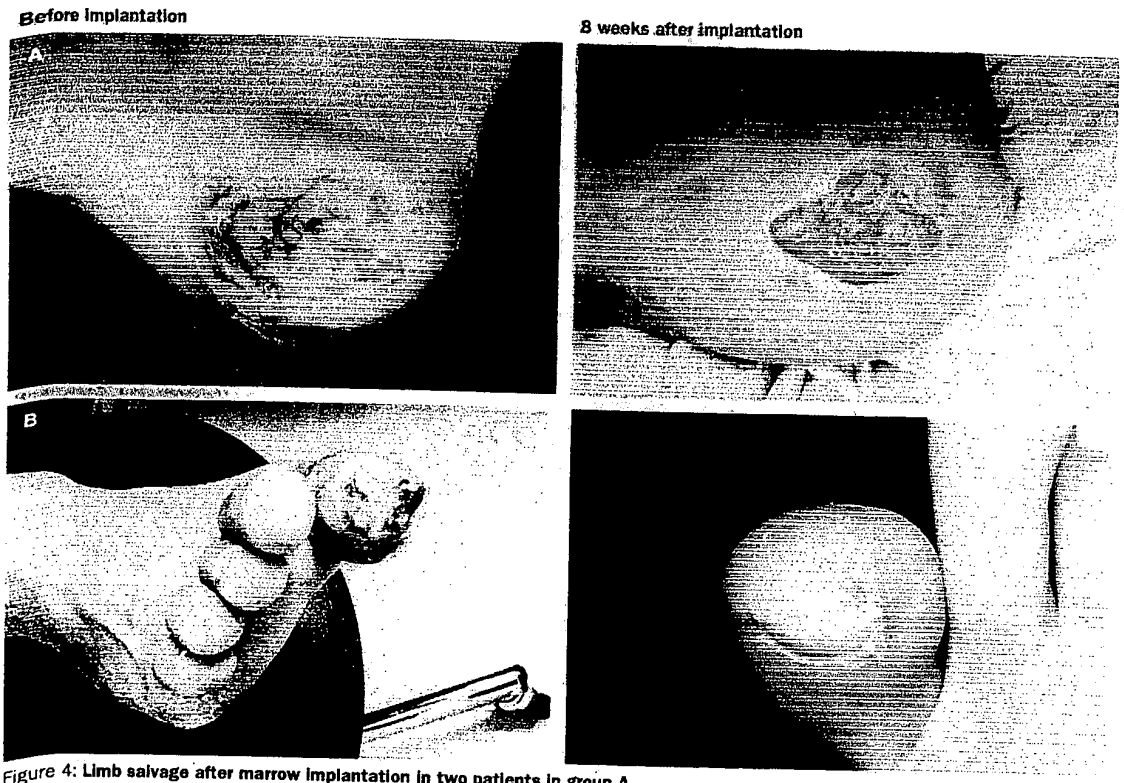

Figure 4: **Limb salvage after marrow implantation in two patients in group A**
Non-healing ulcer on heel (A) and ischaemic necrosis on big toe (B) showed improvement 8 weeks after implantation.

cleansing the measurement site (10 cm distal to tibial tuberosity on anterior skin surface) with alcohol, we applied the probe, and heated the skin to 43·5°C. When a stable steady-state was achieved, a value expressed in mm Hg was recorded (normal range >60).[17] These measurements were made with patients in the supine position, breathing room air. ABI, TcO_2, and walking time were separately measured three times within 10 days before implantation or during days 28–38 after implantation. Interassay variations in all patients were mean 15·7% (SD 1·2), 9·8% (0·14), and 14·6% (0·13), respectively.

We did digital subtraction angiography 1 week before and 4 weeks after implantation, for which amount of contrast, force of contrast injection, and position of the catheter tip were strictly fixed. Two radiologists and one vascular surgeon, who were masked to treatment group, assessed collateral vessels. New collateral vessel formation was assessed at the time at which contrast flow in the main conducting arteries was most clearly visible. New collateral vessels were assessed as +0 (no collateral development), +1 (slight), +2 (moderate), or +3 (rich).

We extracted RNA from $CD34^+$ or $CD34^-$ fractions of bone marrow-mononuclear cells separated with CD34 magnetic microbeads.[6] We used the RNase protection assay to detect mRNA for bFGF (358 bp) and its receptor (302 bp), VEGF (437 bp) and its type 2 receptor (388 bp), and angiopoietin-1 (550 bp) and its receptor (503 bp).[12,18] We analysed the same amounts of RNA by northern blotting with a U3 rRNA probe as a internal standard.[17]

Statistical analysis

Changes in variables from baseline to week 4 or week 24 and differences between active and control treatment were analysed with paired *t* test. All p values were two-tailed; 95% CIs were calculated for differences.

Role of the funding source

The sponsors had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Fluorescence activated cell sorting analyses in all patients showed that 18% (SD 3·8) of $CD34^+$ cells had the characteristic function of endothelial lineage cells (figure 2).

Since we used bone marrow-mononuclear cells containing $CD34^+$ and $CD34^-$ cells, we postulated that these cell fractions might release angiogenic factors to enhance angiogenesis in addition to supply of endothelial progenitor cells. $CD34^-$ cells expressed mRNAs of bFGF much more than VEGF, which was expressed more than angiopoietin-1, but they did not express angiopoietin-2, whereas $CD34^+$ cells predominantly expressed their receptors (figure 2). We assessed serum concentrations of VEGF, bFGF, tumour necrosis factor α, interleukin 1β, interleukin 6 and $CD34^+$ cell numbers immediately before, 12 h, 24 h, and 48 h after marrow implantation. Only interleukin 6 increased from a mean of 6·0 units (SD 5) at baseline to 28 units (9; 12 h maximum) but reverted to baseline concentration within 48 h in all patients. There was no change in number of $CD34^+$ cells associated with marrow implantation.

In groups A and B, ischaemic status, as assessed by ABI and TcO_2, was well matched between legs implanted with bone marrow-mononuclear cells and peripheral blood-mononuclear cells (table 1).

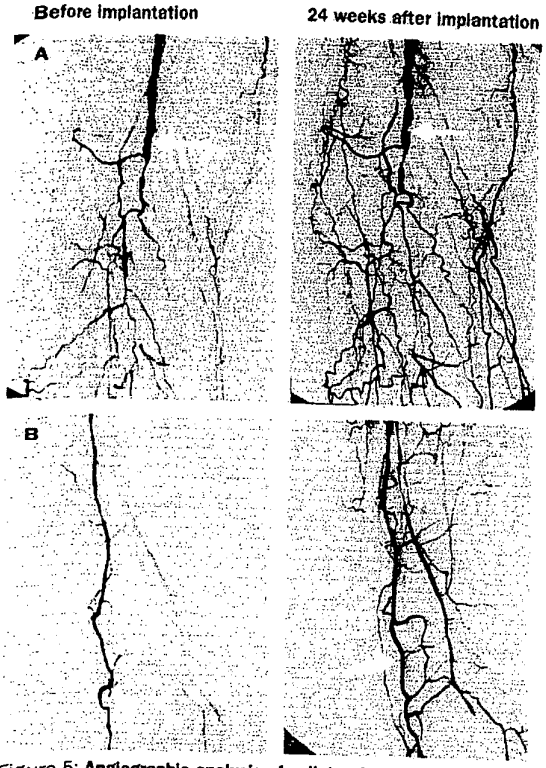

Figure 5: **Angiographic analysis of collateral vessel formation in patients in group A**

Collateral branches were strikingly increased at (A) knee and upper-tibia and (B) lower-tibia, ankle, and foot before and 24 weeks after marrow implantation. Contrast densities in suprafemoral, posterior-tibial, and dorsal pedal arteries (arrows) are similar before and after implantation.

ABI and TcO_2 in legs implanted with bone marrow-mononuclear cells were significantly improved in both groups (figure 3 and table 2). In group A patients, ABI in legs injected with bone marrow-mononuclear cells increased from 0·34 (95% CI 0·27–0·40) at baseline to 0·47 (0·41–0·52) at week 4; increases in ABI (>0·1 at week 4) were noted in 17 of 25 patients. No changes in ABI were seen in legs injected with saline (table 2). ABI values in legs injected with bone marrow-mononuclear cells in group B increased from 0·37 (0·31–0·42) at baseline to 0·46 (0·40–0·52) at week 4 ($p<0·0001$); increases in ABI (>0·1) were noted in 13 of 20 patients. TcO_2 was increased from 28·8 (23–32) at baseline to 46·3 (39–52) at week 4 ($p<0·0001$). By contrast, legs injected with peripheral blood-mononuclear cells showed much smaller increases in ABI and TcO_2 (figure 3 and table 2).

Although rest pain in legs treated with bone marrow-mononuclear cells was resolved in 16 of 20 patients in group B, pain in those treated with peripheral blood-mononuclear cells remained in 17 of 20 patients. Pain-free walking time on treadmill was improved by about 3 min in group A and 1 min in group B (table 2). Diminished improvement in pain-free walking time in group B is probably due to unilateral treatment with bone marrow-mononuclear cells in patients with bilateral limb ischaemia.

Improvement of ischaemic status (ABI, TcO_2, rest-pain scale, pain-free walking time) was maintained during 24-week follow-up (table 2 and figure 3).

In all 45 legs injected with bone marrow-mononuclear cells, ABI values improved from mean 0·35 (95% CI 0·31–0·39) at baseline to 0·42 (0·38–0·46) at week 4 and 0·46 (0·42–0·50) at week 24 ($p<0·0001$). TcO_2 rose from 28 mm Hg (25–31) at baseline to 46 mm Hg (42–50) at week 4 and 45 mm Hg (41–49) at week 24 ($p<0·0001$). Exercise performance was strikingly improved in all patients who were able to do treadmill exercise (n=39; 19 in A, 20 in B). Pain-free walking time increased from 1·3 min (95% CI 1·1–1·5) at baseline to 3·6 min (2·9–4·3) at week 4 and 3·7 min (3·1–4·4) at week 24 ($p<0·0001$). Therapeutic benefit was shown by complete regression of rest pain in 22 patients (12 in A, ten in B), striking improvement of rest pain to pain scale +1 in 15 (nine in A, six in B; table 2), salvage of toe amputation in 15 of 20 patients (eight in A, seven in B), and improvement of ischaemic ulcers in six of ten patients (three in A, three in B; figure 4).

Results of angiography showed a striking increase in number of visible collateral vessels in 27 of 45 patients (15 in A, 12 in B). Representative results of angiography are shown in figure 5. Contrast densities were similar before and 24 weeks after implantation, suggesting that these angiograms were obtained under identical imaging conditions. Laser doppler image analysis in figure 6 shows striking recovery of blood perfusion in the marrow-implanted right leg compared with the contralateral saline-injected leg.

We tested responses of newly formed vessels to prostaglandin by measuring TcO_2 in 30 patients (17 in A, 13 in B) whose ABI was increased by more than 0·1. This pressure before marrow implantation was slightly increased (mean 5·5 mm Hg [SD 6]) in response to prostaglandin infusion (60 μg/2 h), whereas 4 weeks after implantation the pressure rose strikingly (27 mm Hg [13]).

Marrow implantation induced no local inflammatory reaction or oedema of gastrocnemius up to 72 h after implantation. Concentrations of serum creatine phosphokinase increased after implantation (maximum after 1 day), but the maximum range was below 5·3-fold, and reverted to baseline concentration within 7 days. Two patients died of myocardial infarction during 2-year follow-up (both in group A). No adverse events were reported, as judged by the review committees for this trial in the participating university hospitals.

Percutaneous coronary intervention of the right coronary artery of one of the patients in group B who died was done 2·3 months before marrow implantation. Marrow implantation into the ischaemic limb resulted in complete salvage of rest pain. The patient died suddenly of acute myocardial infarction 3·2 months after implantation.

Results of autopsy by pathologists from outside the hospital showed that coronary restenosis was the main pathological finding, and no other lethal lesions were detected. We obtained specimens of the gastrocnemius from the leg that received implantation with bone marrow-mononuclear cells, and analysed vessel formation with anti-CD31 (specific marker for vascular endothelial cells). Vessel numbers assessed by capillary/muscle fibre ratio (2·3 [SD 0·6], ten different fields) were strikingly increased compared with the number in muscle from the contralateral leg that was injected with saline (0·74 [0·31], ten different fields; figure 7).

We noted that $CD31^+$ endothelial cells express Ki-67 in the marrow-implanted limb (figure 7). Ki-67 is a nuclear protein that is expressed in proliferating cells and is scarce in normal vessels.[19] No Ki-67 expression was detected in the saline-injected leg, suggesting presence of proliferating endothelial cells in newly formed vessels. Detailed analysis of leg specimens did not show the presence of bone

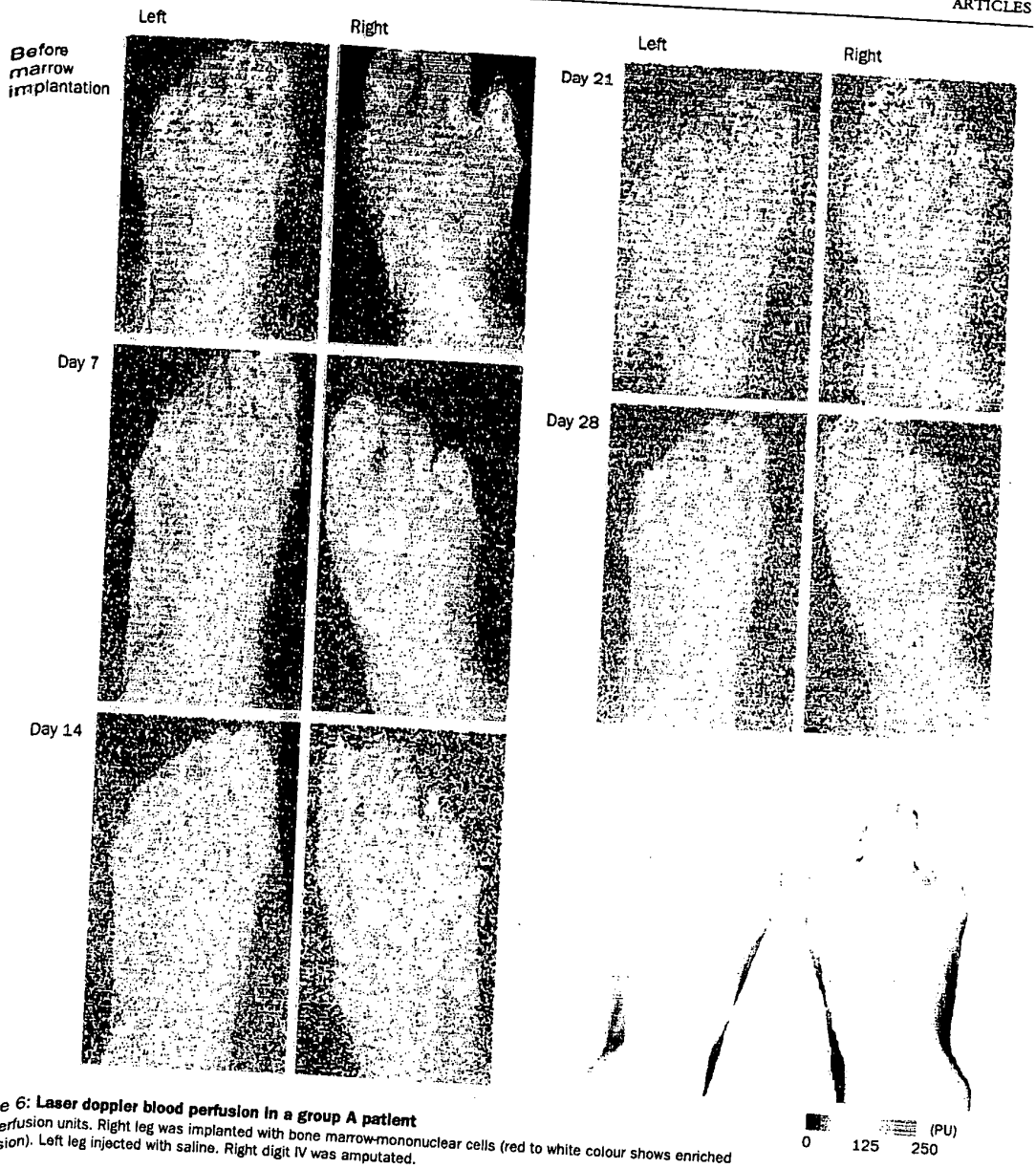

Figure 6: **Laser doppler blood perfusion in a group A patient**
PU=perfusion units. Right leg was implanted with bone marrow-mononuclear cells (red to white colour shows enriched perfusion). Left leg injected with saline. Right digit IV was amputated.

formation by osteoblasts, abnormal fibrosis, or accumulation of inflammatory cells.

Discussion

We have shown here that bone-marrow implantation effectively increased blood flow in all 45 legs, as assessed by substantial increases in ABI, TcO_2, and pain-free walking time or by formation of new collateral vessels formation on angiogram. Implantation of bone marrow-mononuclear cells strikingly improved rest pain in most patients (complete regression in half), and ischaemic ulcers or gangrene were improved in just under half of all limbs, showing successful limb salvage in these legs.

Chronic limb ischaemia is defined as persistently recurring rest pain for more than 2 weeks, or ulceration, gangrene, or both of the foot, with an ankle pressure of 50 mm Hg or greater.[13] Inclusion criteria for our study restricted treatment to patients in whom natural history of critical limb ischaemia had been established for a minimum of 4 weeks of conservative measures without evidence of improvement. In our series of 45 patients, 32 (17 in A, 15 in B) developed limb ischaemia despite having undergone surgical reconstruction before this study.

Pretreatment with granulocyte-colony stimulating factor can increase endothelial progenitor cells in bone marrow or peripheral blood, which could reduce the aspiration volume of marrow cells required or enhance efficacy of collateral vessel formation after implantation of bone marrow-mononuclear cells. In two reports, injection of granulocyte macrophage-colony stimulating factor was noted to

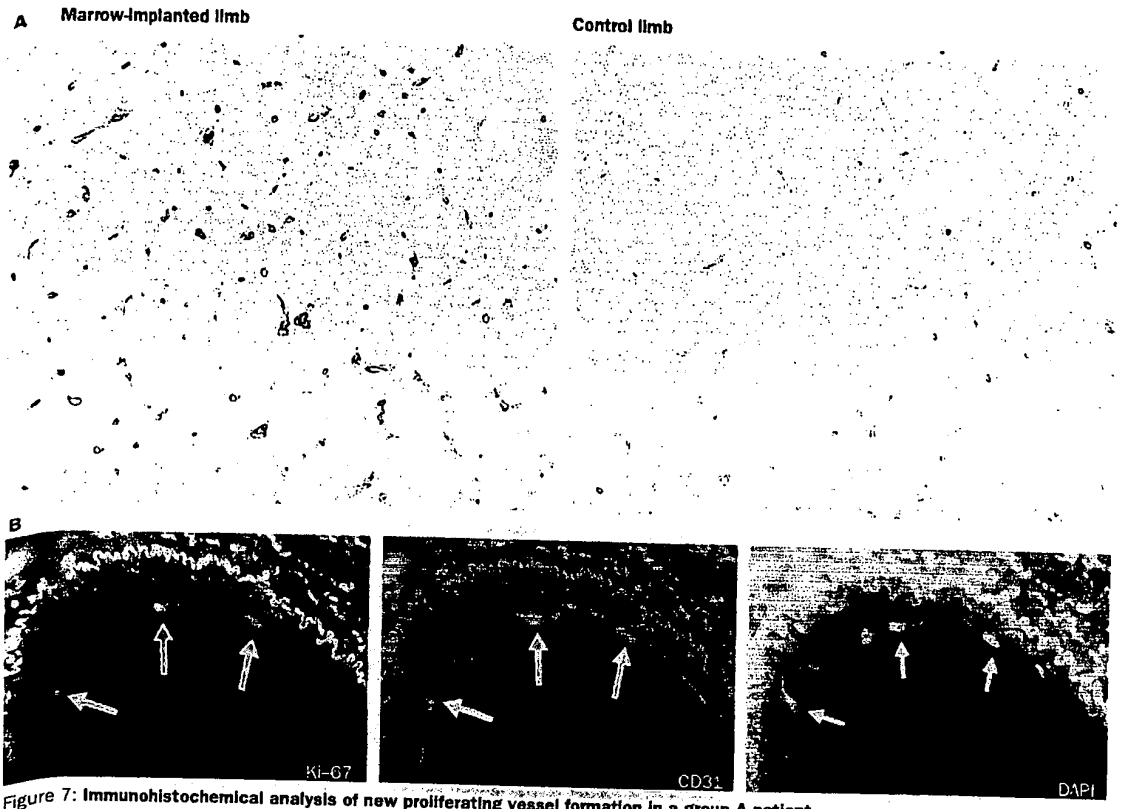

Figure 7: **Immunohistochemical analysis of new proliferating vessel formation in a group A patient**
(A) CD31-positive vessels (brown staining) were strikingly increased in marrow-implanted leg compared with saline-injected control leg (×200). (B) Arrows show endothelial cells positive for Ki-67, CD31, and DAPI (4-6-diamidino-2-phenylindole) (×400).

mobilise endothelial progenitor cells from bone marrow[20] or promote cardiac collateral growth in patients with coronary artery disease.[21] However, we found reports that angina pectoris[22] or acute arterial thrombosis[23] arise in patients receiving granulocyte-colony stimulating factor because of leucocytosis or hypercoagulability. Since most patients eligible for our trial were predicted to have severe atherosclerotic lesions in coronary or cerebral arteries, pretreatment with granulocyte-colony stimulating factor might cause deleterious vascular events before angiogenic cell therapy. We have already reported in rabbits[11] and pigs[12] that intramuscular injection of autologous bone marrow-mononuclear cells effectively enhances collateral vessel formation without pretreatment with granulocyte-colony stimulating factor or granulocyte macrophage-colony stimulating factor. On the basis of these findings, our clinical trial was done without pretreatment with these factors.

When considering clinical potential of therapeutic angiogenesis, it is important to establish whether growth of new capillaries (vasculogenesis) or of pre-existing collateral vessels (angiogenesis) is the therapeutic goal. For newly formed vessels to survive, they must be remodelled and acquire a smooth-muscle coat.[24] In view of the complexity of vessel formation, treatments that use just one angiogenic factor might produce incompletely functioning endothelial channels.[3]

We reported that the $CD34^-$ fraction in bone marrow-mononuclear cells synthesised not only angiogenic growth factors (VEGF and bFGF) but also angiopoietin-1, which is known to have important functions in maturation and maintenance of the vascular system.[25] Takakura and colleagues also reported that marrow haemopoietic cells release angiopoietin-1 to induce maturation of endothelial progenitor cells.[26] Since marrow implantation in our series did not affect circulating concentrations of angiogenic growth factors, combinations of these growth factors might be useful in further treatments directed to neovascularisation of tissues, with adequate enclosure of vessels by periendothelial matrix and pericyte, smooth-muscle cells, or both.

Infusion of endothelial progenitor cells has been shown to induce angiogenesis in ischaemic limbs,[6,7] which accords with our present observation that peripheral blood-mononuclear cells devoid of endothelial progenitor cells had weaker angiogenic activity. Thus, we conclude that efficacy of implantation of bone marrow-mononuclear cells is due to supply of endothelial progenitor cells (included in $CD34^+$ fraction) and multiple angiogenic factors (released from $CD34^-$ fraction). These combined treatments could lead to formation of stable capillary vessels, as suggested by our finding that improvement of limb ischaemia was sustained during 6-month follow-up.

Although results of our unmasked trial (in group A patients) suggest efficacy of implantation of bone marrow-mononuclear cells compared with saline injection, we should be cautious, and take into account the results of our randomised, double-blind trial (in group B patients). Although our results are promising, their interpretation is limited by the small numbers of patients (n=45). Larger trials will be needed to fully understand the implications of our findings.

Since marrow cells include cells of various lineages, such as fibroblasts, osteoblasts, myogenic cells, and endothelial cells, such mixed populations could differentiate into various mesenchymal cells.[8] We have shown in animals that injected bone-marrow mononuclear cells are unlikely to have the ability to differentiate into other lineage cells such as fibroblasts, osteoblasts, and myogenic cells in ischaemic tissues.[11,12] In our study, we immunohistochemically investigated marrow-implanted limbs. Apparent increases in capillary numbers were noted, whereas neither bone formation nor increased interstitial fibrosis was detected. Thus, in ischaemic limbs, endothelial-lineage cells can effectively differentiate into mature cells, whereas some survival factors to stabilise other marrow-derived lineage cells could be lacking in ischaemic conditions.

In summary, we have shown the efficacy and safety of implantation of bone marrow-mononuclear cells in 45 ischaemic limbs. Legs that were injected with peripheral blood-mononuclear cells showed much smaller increases in collateral perfusion. On the basis of criteria proposed by Rutherford and colleagues,[16] limb status was improved in 39 of 45 patients, of whom 30 showed an increase of more than 0·1 in ABI. Thus, autologous implantation of bone marrow-mononuclear cells could constitute a safe and effective strategy for achievement of therapeutic angiogenesis.

Contributors

E Tateishi-Yuyama, H Matsubara, and T Murohara contributed equally to the study. H Matsubara and T Murohara had the original idea for the protocol. H Masaki, T Murohara, and U Ikeda were chief investigators in participating university hospitals and enrolled patients.
E Tateishi-Yuyama, K Amano, Y Kishimoto, S Shintani, K Yoshimoto, and H Akashi did interventions, cared for patients, and did data analysis.
T Iwasaka, T Imaizumi, and K Shimada supervised the study.
H Matsubara contributed to statistical analysis and analysed the results.
The article was revised and approved by all contributors.

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Conflict of interest statement

None declared.

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